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Survival of North American Genotypes of *Trichinella* in Frozen Pork

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ABSTRACT

North American genotypes of *Trichinella spiralis* (T-1), *Trichinella nativa* (T-2), *Trichinella pseudospiralis* (T-4), *Trichinella murrelli* (T-5), and *Trichinella* T-6 were examined for susceptibility to freezing in pork using time-temperature combinations that have been proven to inactivate *T. spiralis*. Infections were established in 3-month-old pigs of mixed sex and breed by oral inoculation of 10,000 muscle larvae (ML) (all genotypes, rodent-derived ML), 20,000 ML (T-1, T-4, and T-5; cat-derived ML), or 30,000 ML (T-2 and T-6; cat-derived ML). Pigs were euthanized 60 days postinoculation. Muscles from the tongue, masseter muscles, diaphragm, triceps, hams, neck, rump, and loins were ground, pooled, and mixed to ensure even distribution of larvae. Samples (20 g) containing each *Trichinella* species, genotype, and source combination were placed in heat-sealable pouches, transferred to a constant temperature refrigerant bath, and maintained according to defined time and temperature combinations. Larvae recovered from cold-treated pork samples were inoculated into mice to determine infectivity. Results indicated that the time-temperature combinations known to render pork safe for *T. spiralis* are sufficient to inactivate *T. nativa* and T-6 (the freeze-resistant isolates), *T. murrelli* (the most common sylvatic species in the United States excluding Alaska), and *T. pseudospiralis* (a species that lacks a muscle nurse cell). These data close a gap in knowledge about the effectiveness of freezing for inactivating these parasites in pork and should alleviate concern about the safety of frozen pork products from the United States.

For much of the 20th century, the consumption of fresh pork in domestic markets and the export of pork and pork products suffered from a negative image derived from the historical presence of *Trichinella* in pigs. During the past 50 years, changes in the pork industry have mostly eliminated *Trichinella* as a risk to consumers of pork from North America and western Europe. However, documentation of pork safety relative to this parasite has been lacking, and the process of gaining consumer confidence in the domestic market and accessing new export markets has been slow.

The United States has relied on two strategies for protecting public health relative to *Trichinella* in pork: education of consumers regarding the need to cook pork and pork products thoroughly and treatment of all ready-to-eat pork products by methods that have been scientifically proven to inactivate the parasite *(15, 16)*. These treatment methods include cooking, curing, and freezing and are described in the U.S. Code of Federal Regulations *(29)*. International market access for frozen pork has been limited, and trading partners may require testing of individual carcasses in addition to cold treatment. In recent negotiations, trading partners agreed to accept frozen pork from the United States as part of the World Trade Organization agreement (http://www.ustr.gov/assets/Document_Library/Fact Sheets/2006/asset_upload_file991_9978.pdf). This agreement was reached in spite of objections raised by international veterinary experts regarding a risk posed by cold-tolerant species of *Trichinella,* some of which are found in the United States and Canada *(8, 22)*. The recent interest in issues surrounding international standards for the cold treatment of pork led members of the International Commission on Trichinellosis to review the current status of the problem and point out the need for additional research on this topic *(25)*.

The research that forms the basis for the current cold treatment requirements for pork *(5, 16, 29)* was conducted in 1990 and was designed to determine the time and temperature combinations that inactivate *Trichinella spiralis* (genotype T-1) in pork. Although the cold-tolerant species *Trichinella nativa* (genotype T-2) was known to exist at the time this research was conducted, its possible role in pig infections and human disease was not considered, and the existence of three other species and genotypes of *Trichinella* found in wildlife in North America, *T. pseudospiralis* (genotype T-4), *T. murrelli* (genotype T-5), and *Trichinella* T-6, was not known. T-6 is resistant to freezing, but the cold tolerance of *T. murrelli* and *T. pseudospiralis* is not clear. Studies suggest that the ability to resist freezing is dependent on a variety of factors, including the host *(19,*

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27). For example, *T. spiralis* shows some tolerance to cold temperature in aberrant species such as the horse *(7)*, and *T. nativa* may be more susceptible to cold treatment in pork *(13)*. However, these preliminary studies require confirmation. The lack of information on how established methods for cold treatment of pork affect species of *Trichinella* other than *T. spiralis* leaves a gap in knowledge that can be used to limit trade or impact public health. A human infection due to the presence of a cold tolerant species or genotype from pork that had been treated by established methods would have serious negative effects on the further use of freezing as a mitigation strategy. For these reasons, a complete understanding of the effect of cold treatment on all relevant species of *Trichinella* is needed to assure that current methods are effective. The purpose of the current study was to fill the current gap in knowledge about susceptibility to cold treatment of sylvatic isolates of *Trichinella* that occur naturally in North America and increase confidence in the cold treatment methods currently used to eliminate the risk of *Trichinella* infection from pork.

MATERIALS AND METHODS

Trichinella species and genotypes occurring in North America were maintained in Swiss-Webster mice and Sprague-Dawley rats at the U.S. Department of Agriculture (USDA), Agricultural Research Service (Beltsville, MD). Each genotype was passaged through experimentally infected domestic cats maintained at the Canadian Food Inspection Agency Centre for Food-borne and Animal Parasitology (CFAP; Saskatoon, Saskatchewan, Canada) to control for possible loss of cold tolerance associated with cycling parasites through rodents instead of carnivorous hosts. Infective muscle larvae (ML) were recovered from mice and rats by artificial digestion *(4)* and then used to inoculate pigs.

Pig infections with North American isolates of *T. spiralis* (genotype T-1), *T. nativa* (genotype T-2), *T. pseudospiralis* (genotype T-4), *T. murrelli* (genotype T-5), and *Trichinella* T-6 that had been maintained in rodents were established in 3-month-old pigs of mixed sex and breed. Three pigs were used for each species and genotype, and each pig was orally inoculated with approximately 10,000 ML. *Trichinella* isolates were initially derived from North American wildlife: *T. spiralis* T-1 from a raccoon in Maryland, *T. nativa* T-2 from a black bear (Istituto Superiore di Sanità [ISS] code 1552; International *Trichinella* Reference Center, http://www.iss.it/site/trichinella/), *T. pseudospiralis* T-4 from a black vulture (ISS code 470), *T. murrelli* T-5 from a coyote (ISS code 1657), and T-6 from a cougar (ISS code 456).

Pig infections with North American isolates that were cycled through domestic cats were also established in 3-month-old pigs of mixed sex and breed. These Canadian wildlife isolates from the CFAP collection were maintained in CD-1 mice prior to passage in cats. T-2 and T-6 were from black bears (R06-44 and R03-07, respectively), and T-4 and T-5 were from cougars (R04-08 and R01-6A, respectively). Two pigs were used for each species and genotype, and each pig was orally inoculated with approximately 20,000 T-1, T-4, or T-5 ML or 30,000 T-2 or T-6 ML.

Infected pigs were euthanized 60 days after inoculation, and muscles from the tongue, masseters, diaphragm, triceps, hams, neck, rump, and loins were collected unilaterally or bilaterally from each pig at necropsy. Muscles were trimmed of excess fat and connective tissue and cut into smaller pieces. Tissue-specific worm burdens were assessed by pepsin-HCl digestion of three 1-g samples from each muscle collected, for a total of 24 g of tissue from each pig *(4)*.

TABLE 1. *Time and temperature combinations prescribed in current USDA regulatory documents (29) for the destruction of* Trichinella spiralis *in frozen pork*

Temp[a]		
°F	°C	Minimum time (h)
0	−17.8	106
−5	−20.6	82
−10	−23.3	63
−15	−26.1	48
−20	−28.9	35
−25	−31.7	22
−30	−34.5	8
−35	−37.2	0.5

[a] Temperature achieved at the center of the meat piece.

Muscle tissue from each pig was individually ground with a commercial meat grinder (model 4612, Hobart Corp., Troy, OH) and then pooled and mixed to assure a uniform distribution of ML in pork samples. Pork samples containing ML derived from rodents were processed and treated separately from pork samples containing cat-derived ML.

To determine tolerance to cold treatment, pork samples containing ML of each species and genotype were cooled according to procedures described by Kotula et al. *(16)*. Samples of pooled blended muscle (20 g each) were packaged in plastic bags (4.5 by 9 in. [11.4 by 22.9 cm]; Whirl-Pak, Nasco, Fort Atkinson, WI) and pressed to a uniform thickness of 2 mm to assure consistency of freezing and thawing rates among samples. Bags were then evacuated of air and heat sealed. For cold tolerance determinations, samples were transferred to programmable constant temperature refrigerant baths (Polystat, Cole Parmer, Vernon Hills, IL) and maintained according to the time and temperature combinations described in Table 1. These time and temperature combinations were based on those listed in current USDA regulatory documents *(29)*. The treatment at −6.6°C (20°F) for 106 h was included as a species-specific positive control to demonstrate that the properly conducted digestion procedure would result in isolation of live worms if present because *T. spiralis* in pork is not killed at this temperature. Five 20-g packaged pork samples for each combination of rodent- or cat-derived inoculum and *Trichinella* species and genotype were tested for each time-temperature combination. A 2-h, 4°C thawing cycle was added to the end of the programmed cold treatment cycle before sample analysis.

All cold-treated samples and a set of untreated positive control samples for each *Trichinella* genotype were digested individually using established methods *(4)*. Cold-treated samples were added to artificial digestion fluid consisting of 1% pepsin and 1% HCl in 1 liter of 45°C tap water. The mixture was held at 45°C with constant stirring for 1 h and then allowed to settle for 20 min. The sediment containing ML was repeatedly washed with 250-ml volumes of tap water and allowed to settle until the supernatant was clear. ML were collected from the sediment and suspended in 1 ml of 0.85% saline warmed to 37°C to increase motility of live ML. The numbers of motile and nonmotile ML were then determined with a stereo microscope at ×40 magnification. All ML (motile and nonmotile) recovered from each 20-g sample were tested for infectivity by oral inoculation into a Swiss-Webster mouse. The maximum inoculation level was 500 ML per mouse. When fewer

than 500 ML were recovered from the five samples from each treatment, all ML were pooled and inoculated into two mice; in no case did any mouse receive more than 500 ML. Thirty-five days postinoculation, mice were euthanized by cervical dislocation, skinned, eviscerated, and digested as described above to isolate and enumerate ML.

One set of five *T. spiralis* samples was treated and digested along with each genotype to assure that appropriate conditions had been used for the destruction of *T. spiralis* ML based on the conditions prescribed in USDA regulations. Each digested sample was examined for recovered ML. All ML (motile and nonmotile) recovered from each 20-g sample were tested for infectivity by oral inoculation into Swiss-Webster mice. Mice were individually digested 35 days postinoculation, and the presence and number of viable ML were determined.

RESULTS

Pigs inoculated with the five mouse-derived *Trichinella* species or genotypes occurring in North American became infected. ML burdens were highest in the predilection sites of the tongue, masseter, diaphragm, and neck muscles (Table 2). Few ML were recovered from pigs infected with 10,000 T-2 or T-6 derived from mice. For T-6, a maximum of 0.4 ML per g was recovered from the most heavily infected tissues (tongue), and for T-2 only three ML total were recovered from the three inoculated pigs; one pig had no ML. Because of the lack of ML, the pork infected with T-2 and T-6 was not subjected to cold treatment. Greater numbers of ML were recovered from pigs given 30,000 T-2 or T-6 ML derived from cats; the number of ML recovered from these pigs was also low (1 to 5 ML per g) when compared with the number of ML recovered from pigs infected with the other species or genotypes. However, sufficient ML were collected to allow these T-2- and T-6-infected pork samples to be used in cold treatment experiments (Table 2).

Pork infected with rodent-derived *T. spiralis, T. pseudospiralis,* and *T. murrelli* and cat-derived *T. spiralis, T. pseudospiralis, T. murrelli, T. nativa,* and T-6 was tested in the cold treatment experiments. Cold treatment results were similar using either rodent- or cat-derived ML. Table 3 shows results from cold treatment of pork infected with rodent-derived *T. spiralis, T. pseudospiralis,* and *T. murrelli* and cat-derived *T. nativa* and T-6 only. Motile and coiled ML were recovered from control (untreated) 20-g packaged pork samples for each genotype tested (Table 4). All mice inoculated with ML isolated from these untreated pork samples became infected, and ML were recovered by digestion from each inoculated mouse at 35 days postinfection.

No motile ML were recovered from any cold-treated sample for any *Trichinella* species tested except for those samples treated at −6.6°C (+20°F). No differences were observed for ML recovered after cold treatment of either rodent-derived or cat-derived ML; all recovered ML were uncoiled and nonmotile. To confirm inactivation, ML recovered from cold-treated samples from each species from each time-temperature combination were inoculated into mice. No ML were recovered from inoculated mice except those inoculated with ML isolated from samples treated at −6.6°C (Table 3).

DISCUSSION

Eight species and four genotypes have been identified in the genus *Trichinella (17, 18, 28)*. Worldwide geographic distribution and host specificity of these isolates has been described *(9, 10, 22, 23, 32)*. Recent studies have shown that five of the sibling species, *T. spiralis* (T-1), *T. nativa* (T-2), *T. pseudospiralis* (T-4), *T. murrelli* (T-5), and *Trichinella* T-6, occur in North America, and two of these sibling species, *T. nativa* and T-6, are capable of surviving for extended periods of time in frozen muscle at temperatures from −5 to −18°C *(6, 8, 20, 26)*.

Human trichinellosis caused by *T. spiralis,* one of the species that is not freeze tolerant, has historically been linked to the consumption of raw or undercooked pork and certain game meats such as bear and wild boar. Much effort has gone into protecting consumers from exposure to *T. spiralis* through carcass inspection, treatment of fresh pork by freezing, cooking, or curing *(29),* and consumer education campaigns regarding proper cooking temperatures to render pork safe *(5, 30)*.

Concerns about the effectiveness of cold treatment (freezing) of pork have been raised given the occurrence of the freeze-tolerant genotypes in North America and the uncharacterized ability of other *Trichinella* species to survive at low temperatures.

Results obtained in the present study indicate that current time-temperature regulations governing treatment of pork products by freezing to inactivate *T. spiralis* are sufficient for inactivation of all *Trichinella* species and genotypes that are found in North America and that might occur in pork.

T. nativa and T-6 both demonstrate significant freeze resistance in the tissues of arctic and subarctic carnivores such as polar bear, grizzly bear, cougar, and arctic fox *(1, 2, 12–14)*. However, previous studies have shown that the freeze resistance of *T. nativa* and T-6 differs depending on the host species in which the larvae are encapsulated, with survival times ranging from hours to years *(11, 13, 31)*. In the present study, *T. pseudospiralis, T. murrelli, T. nativa,* and T-6 encapsulated in the tissues of domestic pigs survived no longer than did *T. spiralis* (an isolate that is not freeze resistant) under the time and temperature conditions tested. Therefore, these species pose little if any risk to consumers of pork that has been treated according to time-temperature freezing guidelines prescribed for inactivation of *T. spiralis* in pork.

The results of the present study confirm previous findings that both *T. nativa* and *Trichinella* T-6 have extremely low infectivity for pigs. *T. nativa* is rarely found in naturally infected pigs or wild boars *(24),* whereas T-6 has never been found in these hosts. Pozio et al. *(25)* reported that the infectivity of *T. nativa* and T-6 for pigs is 10^4 lower than the infectivity of *T. spiralis*. In experimental infections in domestic pigs, low numbers of ML persisted for only a short time in tissues *(8, 11, 21)*. *T. murrelli* and *T. pseudospiralis* both were moderately infective in domestic pigs; however, *T. murrelli* does not persist in swine *(11)*. These data and the susceptibility to freezing indicate that the

TABLE 2. *ML burdens in pig muscles used for cold treatment*

Trichinella genotype (species)	Pig no.	Mean no. of ML recovered[a]							
		Ham	Masseter	Tongue	Diaphragm	Triceps	Rump	Neck	Loin
T-1 (*T. spiralis*)	308[b]	525	875	3,270	801	480	160	1,066	190
	602[b]	33	395	1,033	437	116	145	240	160
	603[b]	233	293	1,120	311	350	200	333	75
	436[c]	186	522	1,558	625	167	66	332	118
	437[c]	82	443	1,327	673	107	95	366	73
T-2 (*T. nativa*)	302[b]	0	0	0	0	0	0	0	0
	304[b]	0	0	0	0	0	2	0	0
	305[b]	0	0	0	0	1	0	0	0
	W32[c]	1	5	6	7	1	1	2	5
	W29[c]	0.3	9	13	8	3	0.3	4	1
T-4 (*T. pseudospiralis*)	553[b]	7	124	245	48	5	29	75	11
	558[b]	26	189	255	122	153	66	88	6
	562[b]	21	144	233	180	88	108	62	42
	W33[c]	24	135	176	209	60	89	33	44
	W28[c]	14	83	331	172	31	33	40	28
T-5 (*T. murrelli*)	303[b]	7	11	37	25	4	7	11	28
	531[b]	1	0	0.3	3	0	0.3	0.6	2
	620[b]	2	0	2	2	0.3	0	0	3
	147[c]	12	34	56	50	14	9	48	24
	146[c]	10	50	90	69	10	7	18	19
T-6 (*Trichinella* sp.)	525[b]	0	0	0	0	0	0	0.3	0.6
	604[b]	0	0	3	2	0	0	0.3	0
	682[b]	0	0.3	0.3	0.3	0.3	0	0	0
	150[c]	18	18	22	11	8	3	4	2
	W30[c]	15	25	24	30	13	8	12	4

[a] Mean value for three 1-g samples.
[b] Pigs inoculated with rodent-derived ML.
[c] Pigs inoculated with cat-derived ML.

sylvatic species of *Trichinella* pose a negligible risk to consumers of pork.

In summary, current regulatory requirements for the treatment of fresh pork products detail specific time and temperature procedures for freezing to inactivate *T. spiralis*. Based on the data from this study, these requirements appear sufficient to inactivate all species and genotypes of *Trichinella* that may occur in pork in North America.

TABLE 3. *Number of ML recovered from cold-treated samples of pork infected with wildlife genotypes of* Trichinella

Storage temp		Storage time (h)	Mean no. of ML recovered[a]				
°F	°C		*T. spiralis*[b]	*T. pseudospiralis*[b]	*T. murrelli*[b]	*T. nativa*[c]	*Trichinella* sp. (T-6)[c]
+20	−6.6	106	4,033	191	245	17	73
0	−17.7	106	548	36	59	0	26
−5	−20.5	82	1,826.4	22	80	12	27
−10	−23.3	63	2,822	7	41	16	32
−15	−26.1	48	2,453	8	54	10	40
−20	−28.8	35	733	61	66	14	36
−25	−31.6	22	199.8	35	39	13	22
−30	−34.4	8	286.40	49	77	16	26
−35	−37.2	0.5	1,112.6	5	26	10	20

[a] Five 20-g pork samples were digested to derive the mean number of ML recovered under specific storage time and temperature conditions. All mice inoculated with these recovered ML were negative for *Trichinella* by digestion 35 days postinoculation except mice given ML treated at +20°F (−6.6°C).
[b] Rodent derived.
[c] Cat derived.

TABLE 4. *Number of ML recovered from control (untreated) pork samples infected with rodent-derived and cat-derived species of* Trichinella *and from mice inoculated with ML from the control pork samples*

Trichinella genotype (species)	Rodent-derived ML		Cat-derived ML	
	ML from pork ($\bar{x}$ ± SD)[a]	ML from mice ($\bar{x}$)[b]	ML from pork ($\bar{x}$ ± SD)[a]	ML from mice ($\bar{x}$)[b]
T-1 (*T. spiralis*)	4,303 ± 144	22,666	9,108 ± 838	18,668
T-2 (*T. nativa*)	ND[c]	ND	18 ± 4	756[d]
T-4 (*T. pseudospiralis*)	188 ± 20.5	7,142	475 ± 120	(13,012)
T-5 (*T. murrelli*)	233 ± 9.7	2,045	138 ± 43	6,567
T-6 (*Trichinella* sp.)	ND	ND	57 ± 13	670[d]

[a] Five 20-g control (untreated) pork samples were digested to derive the mean number of ML recovered.
[b] Five mice were digested to derive mean number of ML recovered.
[c] ND, not done.
[d] When fewer than 500 total ML were recovered from five pork samples, recovered ML were pooled and inoculated into only two mice, which were digested to derive the mean number of ML recovered.

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